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Effect of poly-β-hydroxybutyrate on Chinese mitten crab, *Eriocheir sinensis*, larvae challenged with pathogenic *Vibrio anguillarum*

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Abstract

This study investigated the protective effect of poly-β-hydroxybutyrate (PHB) on Chinese mitten crab, *Eriocheir sinensis,* zoea larvae challenged with pathogenic *Vibrio anguillarum*. PHB was delivered to the crab larvae through rotifer and *Artemia* bioencapsulation. Zoea 3 larvae were challenged with *V. anguillarum* at a final concentration of 10^5 CFU mL^{-1}. PHB-enriched rotifers and *Artemia* nauplii were added to the culture water 24 h prior to, upon and 24 h after challenge. The results confirmed that PHB could enhance the survival and growth of unexposed *E. sinensis* larvae. Moreover, PHB protected larvae from the pathogen as the larvae fed PHB-enriched live food showed the highest survival and development rate in all challenged groups ($P < 0.05$). Furthermore, larval performance was the best when PHB was delivered to the larvae 24 h before challenge ($P < 0.05$). In conclusion, our results indicate that PHB can be used as part of an effective strategy to protect *E. sinensis* larvae from *V. anguillarum* resulting in higher survival and better growth, especially when applied before the challenge.

Keywords: *Artemia* nauplii, *Eriocheir sinensis* larvae, larval development, poly-β-hydroxybutyrate (PHB), survival, *Vibrio anguillarum*.

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Introduction

Infections caused by bacteria are a significant constraint to the development of the aquaculture industry. In the past, antibiotics have been used as therapeutic as well as prophylactic treatments to control bacterial proliferation in aquaculture facilities (Le, Munekage & Kato 2005). However, the widespread use of antibiotics has been extensively criticized as a possible cause of the rapid development of antibiotic resistance in cultured animals, consequently posing threats to human health as well as the environment (Cabello 2006; Sapkota *et al.* 2008). Therefore, new strategies to control bacterial infections are urgently needed to make aquaculture development more sustainable.

Short-chain fatty acids (SCFAs) are considered alternatives to antibiotics as they inhibit the growth of pathogenic bacteria (usually Gram-negative bacteria) rather than killing them and thus entailing less selective pressure for the development of resistance (Defoirdt *et al.* 2007a). Some promising results have been obtained when supplementing SCFAs in livestock and poultry feeds, indicating that SCFAs inhibit the growth of enterobacteria (Cherrington *et al.* 1991; Sun *et al.* 1998; Van Immerseel *et al.* 2003). *In vivo* challenge tests with the aquaculture model organism *Artemia* showed that formic, acetic, propionic, butyric and valeric acid inhibited the growth of the luminescent pathogenic *Vibrio campbellii* under gnotobiotic conditions. Moreover addition of SCFAs to the culture medium significantly increased the survival of *Artemia* nauplii challenged with *V. campbellii* (Defoirdt *et al.* 2006). The application of SCFAs in

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aquaculture is, however, difficult because of its water solubility and the high effective concentrations required (Defoirdt *et al.* 2006).

Poly-β-hydroxybutyrate (PHB) is the polymer of β-hydroxybutyric acid. It can be degraded into oligomers and monomers in the presence of enzymes or under acidic/alkaline conditions (Yu, Plackett & Chen 2005). It is assumed that PHB polymers are at least partially degraded into water-soluble β-hydroxybutyrate monomers in the gut of *Artemia*, which could have antibacterial activity similar to the SCFAs (Defoirdt *et al.* 2007b). PHB, both in the form of particles and in the form of PHB accumulating bacteria, has been shown to significantly increase the survival of *Artemia* when challenged with *V. campbellii* under both gnotobiotic and conventional conditions (Defoirdt *et al.* 2007b; Halet *et al.* 2007). Recent studies have shown that feeding PHB-enriched *Artemia* can improve the growth as well as reduce the total amount of bacteria and *Vibrio* inside *Macrobrachium rosenbergii* larvae (Nhan *et al.* 2010). Dietary supplementation with 2% and 5% PHB had a beneficial effect on growth performance and on the intestinal bacterial community structure of European seabass, *Dicentrarchus labrax* (L.), juveniles (De Schryver *et al.* 2010).

Chinese mitten crab, *Eriocheir sinensis,* larval rearing and farming are widely practiced in China (Sui *et al.* 2011). However, the intensive larviculture techniques applied in hatcheries usually result in a rapid rise in pathogen levels in the rearing water, which may become deleterious to the larvae. Vibrios such as *V. parahaemolyticus, V. anguillarum* and *V. alginolyticus* are often detected in local mitten crab hatcheries and farms (Lu *et al.* 1999; Xu *et al.* 2002). Larvae usually suffer from damage to the spine and low appetite, and consequently, high mortalities are observed when infected with vibrios (Li *et al.* 2001). In the present study, the protective effect of rotifer or *Artemia*-bioencapsulated dietary PHB administration on *E. sinensis* larvae challenged with the virulent pathogen *V. anguillarum* was investigated. The results provided practical information on the use of PHB as biocontrol strategy in commercial crab larviculture.

Materials and methods

Larval rearing

The experiment was conducted in May 2010. A wild berried female crab was obtained from the Haihe river estuary in China. The procedures of egg incubation and larval hatching were similar to those described in Sui *et al.* (2007). In brief, the berried female was incubated in saline water (20 g L^{-1}) for 14 days by gradually increasing temperature from 15 to 21 °C at a rate of 0.5–1 °C per day until the larvae hatched. Larvae were then reared at 22–23 °C and fed *ad libitum Chlorella* and rotifers at the zoea 1 (Z1) and Z2 stages, respectively. Upon moulting of Z2 into Z3 at DAH6 (6 days after hatching), groups of 100 active-swimming Z3 were collected and transferred to plastic beakers containing 1.5 L seawater and then reared at 23–24 °C until all Z5 had metamorphosed to megalopa. Water was renewed every day or every other day. Rotifers and newly hatched *Artemia* nauplii were fed to crab larvae. The applied prey density was as follows: Z3: 10 rotifers + 0.5 *Artemia* mL^{-1}; Z4: 1 *Artemia* mL^{-1}; Z5: 1.5 *Artemia* mL^{-1}. Larvae were fed three times a day at 9:00 h, 14:00 h and 22:00 h. Each treatment had four replicates.

PHB enrichment

Rotifers, *Branchionus plicatilis,* were cultured with frozen *Chlorella* concentrate, and *Artemia* cysts (from Aibi Lake, China) were hatched using standard hatching conditions (Lavens & Sorgeloos 1996). Both rotifers and newly hatched *Artemia* nauplii were enriched with commercial PHB particles (size range 10–50 μm, Good Fellow) at a dose of 100 mg L^{-1}. The density of rotifers and *Artemia* during enrichment was kept at 2000 mL^{-1} and 300 individuals mL^{-1}, respectively. Enrichment was done for 24 h at a water temperature of 28 °C. After enrichment, the live food organisms were rinsed thoroughly with clean saline water (20 g L^{-1}) to remove any remaining PHB before being fed to the larvae (Sun & Sui 2011).

Preparation of the inocula for *in vivo* challenge test

A pathogenic strain of *V. anguillarum* was obtained from the Bacteria Collection of the Institute of Oceanography, Chinese Academy of Sciences. It was preserved in 40% glycerol at −80 °C. Before use, the strain was inoculated on sterile marine nutrient agar and incubated at 28 °C for 24 h. After three rounds of subculturing, a colony was picked up from the agar plate and inoculated into a beaker containing sterile marine nutrient broth.

The beaker was incubated on a horizontal shaker at 28 °C overnight for 9–12 h to ensure the bacteria were at the logarithmic growth phase. Prior to the challenge test, the optical density at 600 nm of the *V. anguillarum* suspension was measured with a spectrophotometer, which was then converted into the bacterial density according to a colony–optical density standard curve previously obtained under the same culture conditions.

Experimental design

Six treatments were conducted to investigate the protective effect of PHB on *V. anguillarum* challenged *E. sinensis* larvae (Table 1). Different feeding regimes were applied to two groups of *E. sinensis* larvae, referred to as unchallenged control groups (C) and *V. anguillarum* challenged groups (T), respectively. For the *in vivo* challenge test, Z3 larvae with a density of 100 individuals per 1.5 L^{-1} were challenged by directly adding *V. anguillarum* suspension (10^8 CFU mL^{-1}) into the rearing water to a final density of 10^5 CFU mL^{-1}.

Evaluation parameters

To evaluate the effect of PHB on *V. anguillarum* challenged *E. sinensis* larvae, the survival and larval stage index of different treatments were determined every other day or every day when rearing water was renewed. Survival percentage was calculated on the initial number of Z3. To avoid cannibalism on the remaining Z5, newly metamorphosed megalopa were transferred daily to new beakers and fed according to their respective feeding treatment. Larval development was calculated as the average larval stage in each treatment and expressed as larval stage index (LSI) as described by Millamena & Bangcaya (2001).

Statistical analysis

Survival and larval stage index of the crab larvae fed with various feeding regimes were subjected to the software SPSS (version 17.0, 15.0, SPSS Inc.) for statistical analysis. Statistically significant differences amongst treatments were determined using one-way ANOVA and comparison with Tukey as a post hoc test. Data were regarded as significantly different when $P < 0.05$.

Results

Survival of *E. sinensis* larvae subjected to the different feeding treatments is shown in Table 2. Compared with the challenged groups, better survivals were observed in the unexposed control groups fed PHB-enriched (C2) and non-enriched live food (C1) (47.5% and 33.8% at DAH 16, respectively) ($P < 0.05$). PHB-enriched live food feeding resulted in a significantly higher survival of unexposed larvae from DAH 12 onwards ($P < 0.05$). Amongst the challenged groups, the survival of larvae fed PHB-enriched live food (T1, T2 and T3) was significantly higher than that of larvae fed non-enriched live food (C3) ($P < 0.05$). Moreover, when PHB-enriched live food was delivered 24 h before the challenge, a higher larval survival was obtained. The average survival of the T1, T2 and T3 groups (shift from non-enriched to PHB-enriched live food 24 h before, simultaneously with and 24 h after challenge, respectively) at DAH 16 were significantly different from each other (26.3%, 18.8% and 11.9%, respectively), whilst survival in the C3 group was close to zero (0.6%, $P < 0.05$).

Larval development rates in the different treatments are summarized in Table 3. For the

Table 1 Experimental feeding regimes used for *Eriocheir sinensis* larvae

Treatments	C1	C2	C3	T1	T2	T3
Challenge with Vibrio anguillarum	Not challenged		Challenged			
Live food	Non-enriched	PHB-enriched	Non-enriched	PHB-enriched	PHB-enriched	PHB-enriched
Time of change from non-enriched to PHB-enriched live food	No	No	No	24 h before challenge	Simultaneously with challenge	24 h after challenge

*C1 – unchallenged larvae, fed non-enriched live food; C2 – unchallenged larvae, fed PHB-enriched live food; C3 – *V. anguillarum* challenged larvae fed non-enriched live food; T1 – *V. anguillarum* challenged larvae, changed from non-enriched to PHB-enriched live food feeding 24 h before challenge; T2 – *V. anguillarum* challenged larvae, changed from non-enriched to PHB-enriched live food feeding upon challenge; T3 – *V. anguillarum* challenged larvae, changed from non-enriched to PHB-enriched live food feeding 24 h after challenge.

Table 2 Survival of *Vibrio anguillarum* challenged *Eriocheir sinensis* larvae subjected to different feeding regimes

	Survival percentage (%)					
Treatments	DAH8	DAH10	DAH12	DAH14	DAH15	DAH16
C1	81.3 ± 1.4^{ab}	68.8 ± 4.3^{ab}	58.8 ± 3.2^{b}	55.6 ± 3.1^{b}	37.5 ± 2.0^{b}	33.8 ± 1.4^{b}
C2	85.6 ± 1.3^{a}	73.1 ± 4.7^{a}	66.3 ± 3.2^{a}	65.6 ± 2.4^{a}	48.1 ± 1.3^{a}	47.5 ± 2.0^{a}
C3	51.9 ± 2.4^{d}	39.4 ± 5.2^{d}	31.9 ± 2.4^{e}	18.8 ± 1.4^{f}	6.3 ± 1.4^{f}	0.6 ± 1.3^{f}
T1	76.9 ± 3.8^{b}	60.6 ± 5.2^{bc}	53.1 ± 1.3^{c}	49.4 ± 2.4^{c}	29.4 ± 1.3^{c}	26.3 ± 1.4^{c}
T2	65.0 ± 2.0^{c}	55.6 ± 5.2^{c}	46.9 ± 1.3^{d}	38.8 ± 1.4^{d}	20.6 ± 1.3^{d}	18.8 ± 1.4^{d}
T3	63.1 ± 3.1^{c}	54.4 ± 2.4^{c}	44.4 ± 2.4^{d}	31.3 ± 2.5^{e}	14.4 ± 1.3^{e}	11.9 ± 1.3^{e}

Data are shown as mean ± standard deviation ($n = 4$). Values in the same column showing a different superscript letter are significantly different ($P < 0.05$). Refer to Table 1 for treatment description.

Table 3 Larval stage index (LSI) values of *Vibrio anguillarum* challenged *Eriocheir sinensis* larvae subjected to different feeding schemes

	Larval stage index (LSI)					
Treatments	DAH9	DAH10	DAH12	DAH14	DAH15	DAH16
C1	3.77 ± 0.07^{ab}	3.94 ± 0.02^{ab}	4.76 ± 0.04^{ab}	5.00 ± 0.04^{ab}	5.53 ± 0.03^{b}	6.00
C2	3.81 ± 0.04^{a}	3.96 ± 0.01^{a}	4.80 ± 0.03^{a}	5.05 ± 0.04^{a}	5.86 ± 0.03^{a}	6.00
C3	3.25 ± 0.05^{c}	3.88 ± 0.04^{b}	4.53 ± 0.04^{d}	4.83 ± 0.06^{d}	5.00 ± 0.00^{d}	6.00
T1	3.71 ± 0.08^{ab}	3.93 ± 0.02^{ab}	4.79 ± 0.05^{a}	4.99 ± 0.03^{ab}	5.53 ± 0.04^{b}	6.00
T2	3.70 ± 0.05^{ab}	3.92 ± 0.03^{ab}	4.68 ± 0.04^{bc}	4.94 ± 0.05^{bc}	5.49 ± 0.03^{b}	6.00
T3	3.67 ± 0.03^{b}	3.91 ± 0.04^{ab}	4.61 ± 0.03^{cd}	4.88 ± 0.04^{cd}	5.30 ± 0.07^{c}	6.00

Data are shown as mean ± standard deviation ($n = 4$). Values in the same column showing a different superscript letter are significantly different ($P < 0.05$). Refer to Table 1 for treatment description.

unexposed control groups, the LSI in the C2 group (fed PHB-enriched live food) was generally higher than for the C1 group (fed unenriched live food), although this only became statistically significant at DAH 15 when most Z5 had metamorphosed into megalopa ($P < 0.05$). In the challenged groups, a significantly lower LSI was observed in the C3 group (fed with non-enriched live food) for most of the sampling points ($P < 0.05$). On the other hand, no significant difference was observed between T1 and T2 at DAH9, DAH10 and DAH15 ($P < 0.05$), whilst the LSI of the T3 group was between these two groups.

Discussion

In this study, we investigated the possibility of application of the bacterial storage compound PHB as a biocontrol compound in mitten crab larviculture. Compared with juveniles and adults, mitten crab zoea larvae are more susceptible to *Vibrio* infection (Li *et al.* 2001). During the experiment, we observed reddish colouration of appendages, broken dorsal spines and empty digestive tracts in the groups challenged with *V. anguillarum*, which are the signs of *Vibrio* infected zoea larvae (Yu X.Q., personal communication). Two days after challenge, the survival of challenged larvae fed unenriched live food (C3, 51.9% at DAH8) was 30% less than in the unchallenged group fed unenriched live food (C1, 81.3% at DAH8), demonstrating that this particular *Vibrio* strain when applied under the described conditions (10^5 CFU mL^{-1}) is pathogenic to mitten crab zoea larvae.

Our previous studies indicated that PHB with a particle size of < 50 μm can be effectively bioencapsulated into rotifers and *Artemia* nauplii at a dose of 100 mg PHB L^{-1} (Sun & Sui 2011) and PHB-enriched live food improved the overall performance of *E. sinensis* larvae and enhanced the food conversion rate (unpublished data). Similar results were also observed in *M. rosenbergii* larviculture (Nhan *et al.* 2010). The current results confirm that PHB has significantly positive effects on the survival and larval development of unchallenged *E. sinensis* larvae. This is thought to relate to the degradation of PHB polymers into β-hydroxybutyric acid monomers inside the larval gut (Defoirdt *et al.* 2007a) affecting the gut pH. SCFAs, particularly β-hydroxybutyric acid, are known to be a preferred energy source for *Artemia* (Weltzien

et al. 2000). Moreover, the reduction in the gut pH may improve the activity of digestive enzymes, thus inducing better digestibility and absorption of nutrients from the feed (Baruah *et al.* 2005, 2006).

The current study showed that PHB can help protect *E. sinensis* larvae from pathogenic *V. anguillarum*. Similar results were observed in *Artemia* when challenged with luminescent *V. campbellii* (Defoirdt *et al.* 2007b; Halet *et al.* 2007). Several studies have shown that organic acids can improve disease resistance in fish and crustaceans. Ramli, Heindle & Sunanto (2005) reported that hybrid tilapia, *Oreochromis* sp., fed diets supplemented with potassium diformate had significantly better weight gain, feed utilization efficiency and survival when challenged with *V. anguillarum*. The use of organic acids and salt resulted in better survival of tilapia during challenge with *Streptococcus agalactiae* (Ng *et al.* 2009). Small, undissociated fatty acid molecules can pass through the cell membrane of the Gram-negative bacteria and release protons in the cytoplasma (Cherrington *et al.* 1991). The cells therefore need to consume energy to keep their pH at the optimal level, inhibiting bacterial growth. Therefore, we assume that the release of β-hydroxybutyric acid in the larval gut may protect *E. sinensis* larvae from the pathogen.

Furthermore, our results indicated that *E. sinensis* larvae benefited from an early exposure to PHB. If PHB was delivered 24 h before the addition of the pathogen, a more pronounced effect was noticed. Apart from suppressing the growth of bacteria, PHB could hypothetically also benefit the host more directly resulting in a higher resistance to the pathogen. Apart from being a potential energy source and enhancing the enzymatic activity in the larval gut, the lower gut pH may also help to maintain a relative stable intestinal microbial ecosystem, favouring the growth of Gram-positive bacteria, including putative probiotic bacteria (Baruah *et al.* 2008).

In conclusion, the results obtained in this study showed that PHB, through live food enrichment, significantly increased larval survival and development of *E. sinensis* when challenged with pathogenic *V. anguillarum*, and earlier delivery of PHB results in better protection of the larvae. In practice, PHB enrichment of live feed as a prophylactic tool seems to have more potential compared with its use as a therapeutic in production conditions.

Further research is needed with respect to the mode of action of PHB including its effect on the microbial community structure and activity of the gut, and direct immunological effects on the larvae.

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